NL-3 K Strain Is a Stable and Naturally Occurring Interspecific Recombinant Derived from *Bean common mosaic necrosis virus* and *Bean common mosaic virus*

Richard C. Larsen, Phillip N. Miklas, Keri L. Druffel, and Stephen D. Wyatt

First and second authors: U.S. Department of Agriculture–Agricultural Research Service, Vegetable and Forage Crop Research Unit, 24106 North Bunn Road, Prosser, WA 99350; third author: Washington State University, Pullman 99164; and fourth author: Washington State University (retired), 1505 Little Kitten, Manhattan, KS 66503.
Accepted for publication 19 April 2005.

ABSTRACT

Larsen, R. C., Miklas, P. N., Druffel, K. L., and Wyatt, S. D. 2005. NL-3 K strain is a stable and naturally occurring interspecific recombinant derived from *Bean common mosaic necrosis virus* and *Bean common mosaic virus*. Phytopathology 95:1037-1042.

A strain of *Bean common mosaic necrosis virus* (BCMNV) from Idaho was identified by enzyme-linked immunosorbent assay using monoclonal antibodies and determined to be similar to the NL-3 D strain (of Drijfhout) by reaction of differential bean cultivars. However, this BCMNV strain (designated NL-3 K) caused earlier and more severe symptoms on bean plants representing host groups 0, 4, and 5. The nucleotide sequence encoding the predicted polyprotein of NL-3 K was 9,893 nucleotides (nt) in length, yielding a peptide with a molecular size of 362.1 kDa compared with a 9,626-nt, 350.9-kDa polyprotein for NL-3 D. Sequence analysis of the putative P1 protein suggests that the NL-3 K strain is a recombinant between NL-3 D and the Russian strain (RU1) of *Bean common mosaic virus.* The P1 protein of NL-3 K consisted of 415 amino acids compared with 317 for NL-3 D. The first 114 predicted amino acids of the NL-3 K P1 region were 98% identical with RU1. The remaining 301 amino acids of the protein shared only 34% identity with RU1 but were 98% identical with NL-3 D. Primers were designed that flanked the recombination point in the P1 coding sequence of NL-3 K. An amplicon of the expected size was produced by reverse-transcriptase polymerase chain reaction of total nucleic acid extracts of bean plants inoculated with NL-3 K, but not from those with NL-3 D or RU1. The increased symptom severity on selected common bean lines induced by NL-3 K suggests that the P1 gene may play a significant role in pathogenicity and virulence.

Additional keywords: pathogenicity gene, *Phaseolus vulgaris*, *Potyviridae*, resistance genes.

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are species of the family *Potyviridae*. Designated strains of each virus originally were identified and assigned to serogroup A (BCMNV) or serogroup B (BCMV) by Drijfhout (6). All strains of BCMNV were designated as BCMV prior to 1992, at which time serogroup A strains were demonstrated to be distinct from those in serogroup B based on serology, molecular analyses, and ultrastructural comparisons (14, 15,23,39). Additional serological assays and nucleotide sequence analysis indicated that other virus strains previously considered distinct viruses, including *Azuki bean mosaic virus*, *Blackeye cowpea mosaic virus* (BlCPMV), *Dendrobium mosaic virus*, and *Peanut stripe virus* (PStV), cluster with BCMV, whereas *Cowpea aphidborne mosaic virus* and *South African passiflora virus* cluster with BCMNV (3,14). At least 19 different strains of BCMV have been identified and biologically authenticated. Only four strains (TN-1, NL-3, NL-5, and NL-8) have been identified for BCMNV (3,6,11,15). All known strains of each virus are transmitted by several aphid species in a nonpersistent manner and both viruses are seedborne. The viruses occur nearly worldwide and historically have been responsible for serious yield losses in common bean (*Phaseolus vulgaris* L.).

Whether strain evolution within BCMV and BCMNV is due mainly to mutation or RNA recombination between different strains or viruses is not well defined. A precedent for natural recombination within the family *Potyviridae* was reported by Cervera et al. (4), who demonstrated that a field isolate of *Plum pox virus* was the result of natural recombination between two different strains of the virus that occurred primarily in the 3′ proximal terminus of NIb or the 5′ proximal terminus of the coat protein (CP) gene. Tan et al. (38) analyzed 89 isolates of *Turnip mosaic virus* for evidence of recombination. Eighteen recombination sites were located in the 5′ two-thirds of the genome compared with only two sites in the 3′ one-third. They found that 24 and 35% of the P1 and NIa-VPg gene sequences, respectively, were recombinants, compared with only 1% in the corresponding NIa-Pro and CP gene sequences. Revers et al. (28) evaluated the coat protein and 3′ untranslated regions (UTRs) of 15 strains of BCMV. Statistical analyses and phylogenetic models suggested that BCMV strains NL-4 and PStV-1b were possible recombinants. A subsequent study identified specific regions in the CP gene and the 3′ UTR that supported NL-4 as a recombinant strain (24). Silbernagel et al. (34) hypothesized that recombination among strains of BCMV and BCMNV was possible based on phenotype. Interestingly, five of the putative recombinant strains were produced following co-inoculation with BCMNV NL-8 and BCMV US-5 and phenotypically resembled the naturally occurring Russian strain. Mixed infections of BCMV and BCMNV isolates have been observed under natural field conditions in surveys conducted in Africa (P. N. Miklas and R. C. Larsen, *unpublished data*; 35), Mexico (8), and Spain (30). Reports of recombination have focused on the CP genes and 3′ UTRs of viral strains or isolates within genera of the family *Potyviridae* (4,25,26,28,32), although recent studies have demonstrated recombination in the

Corresponding author: R. C. Larsen; E-mail address: rlarsen@pars.ars.usda.gov

DOI: 10.1094/PHYTO-95-1037

helper component-proteinase (HC-Pro) and P1 regions (5,9,36). Spence and Walkey (35) and Silbernagel et al. (34) have detected numerous BCMV and BCMNV strains with novel pathogenic phenotypes using a panel of dry bean cultivars. These unusual strains may represent recombinants or mutants.

Recently, a highly virulent strain of BCMNV NL-3 was discovered in Kimberly, ID, and designated NL-3 K. Strains NL-3 D and NL-3 K both reacted similarly across a set of bean host group differentials used to identify specific strains of BCMV and BCMNV, but responses were different across selected genotypes (21,37). Characteristics of NL-3 K infection included more severe symptoms with earlier onset. Enzyme-linked immunosorbent assay (ELISA) tests verified the virus as a strain of BCMNV and that the virus culture was not a mixed infection with any other known strain of BCMV. The objective of this research was to identify significant differences in the genome of NL-3 K that may be responsible for the pathogenic phenotype of this unusual BCMNV strain.

MATERIALS AND METHODS

Host inoculations and serology. A set of bean differentials representative of each of 12 host groups (HGs) (6) were evaluated for reaction to infection with BCMNV NL-3 K and NL-3 D, respectively. The differentials included cvs. Sutter Pink (HG 0), Bountiful (HG 1), Redlands Greenleaf C (HG 2), Redlands Greenleaf B (HG 3), UI-123 (HG 3), UI-34 (HG 4), UI-114-8 (HG 5), UI-31 (HG 6), IVT-7214 (HG 7), Black Turtle 1 (HG 8), Top Crop (HG 9), Jubila (HG 9), Red Kloud (HG 10), US92-1006 (HG 11), and TARS-VR-7s (HG 12). An additional set of bean germplasm lines developed by Miklas et al. (16–19) with resistance to BCMV and BCMNV also were evaluated for resistance to infection with NL-3 K or NL-3 D. Lines USLK-1, USLK-2, USLK-3, USDK-4, USDK-5, USWK-6, USCR-7, and USCR-9 each contained the resistance genes *I* + *bc3*, whereas USCR-8 contained only the *bc3* gene (20). Infected source tissue was macerated in cold 50 mM potassium phosphate, pH 7.4, containing 10 mM sodium sulfite. Plants were mechanically inoculated at the primary leaf stage with NL-3 K or NL-3 D after dusting with 600-mesh carborundum and maintained in the greenhouse at 18 to 30°C. Symptoms on primary leaves were recorded 11 days postinoculation (dpi) and systemic reactions were recorded 19 dpi. All test plants were evaluated by indirect ELISA using the universal potyvirus group-specific monoclonal antibody (MAb) (Agdia, Inc., Elkhart, IN). Additional assays were conducted by double-antibody sandwich ELISA using the BCMNV-specific MAb I-2 (22) and the BCMV-specific MAb B-1-5E5 (39).

Preparation of total nucleic acid and reverse-transcriptase polymerase chain reaction procedure. Total nucleic acid was extracted from bean plants at the trifoliate stage using the method described by Presting et al. (27), except that 100 mg of tissue was macerated in buffer without the use of liquid nitrogen. After washing with 70% ethanol, the final pellet was resuspended in 300 µl of sterile water. Primers for use in reverse-transcriptase polymerase chain reaction (RT-PCR) were designed for BCMNV NL-3 D (GenBank accession no. AY138897) and the Russian strain (RU1) of BCMV (AY863025). Primers NL-3 D forward (5′-CCATTGCTGCTGAGATTC-3′) and NL-3 D reverse (5′- AGTTCACCGTGAGATGTC-3′) amplified a 714-bp fragment located within the P1 region. Primers BCMV-RU1 forward (5′-CACCGTGCCACTTGTATG-3′) and BCMV-RU1 reverse (5′-GCCGATGTATTCCTTCTG-3′) amplified a 710-bp fragment also located in the P1 region of the virus genome. cDNA for RT-PCR was synthesized in 20-µl reactions consisting of 50 mM Tris, pH 8.0; 75 mM potassium chloride; 3 mM magnesium chloride; 5 mM dithiothreitol; 500 µM each dATP, dCTP, dGTP, and dTTP; 10 ng of reverse primer; and 2 µg of total nucleic acid preparation. After 3 min at 70°C followed by cooling to 4°C, 200 units of MMLV RT (Promega Corp., Madison, WI) was added to the reaction and incubated at 42°C for 1 h. The subsequent PCR amplification consisted of 25-µl reactions containing 2 µl of first-strand cDNA template, 10 mM Tris (pH 8.0), 50 mM potassium chloride, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 150 mM each of dATP, dCTP, dGTP, dTTP, 5 ng each of forward and reverse primer, and 1 unit of *Taq* DNA polymerase (Promega Corp.). Thermocycling parameters were optimized and a final profile was employed that consisted of a single cycle of 3 min at 95°C followed by 25 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, followed by a final extension step for 7 min at 72°C. To verify the authenticity of the RT-PCR products, the DNA product was purified from an agarose gel using GeneCapsule (Geno Technology, Inc., St. Louis) and cloned into the vector pCR4 Topo using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA was purified from *Escherichia coli* DH10β using alkaline lysis (31) and clones containing the PCR product were identified by digestion with *Eco*RI and visualized by agarose gel electrophoresis. The cloned DNA products were sequenced using the dideoxy-chain termination method.

Virus purification, cDNA synthesis, and cloning of BCMNV NL-3 K viral genome. The virus was propagated in bean 'Sutter Pink' or UI-34 and harvested for virus purification 12 to 14 dpi using a procedure described by Larsen et al. (13). The partially purified virus was adjusted to cesium chloride at 40 mg/ml (wt/vol) and centrifuged for 15 to 18 h at 125,000 × *g* at 12°C. The light-scattering band was collected, diluted in 16.5 mM Na_2HPO_4 and 1.8 mM sodium citrate, pH 8.0, and centrifuged for 1 h at 250,000 × *g*. The final pellet was resuspended in 10 mM Tris, pH 8.0.

cDNA was synthesized initially from the NL-3 K viral RNA template by RT-PCR utilizing custom primers designed based on available sequence for NL-3 D in GenBank. The RT reactions were conducted as follows. First, 5 µl of viral RNA template and 10 ng of reverse primer were incubated at 70°C for 3 min and chilled at 4°C. Then, 13 µl of RT reaction mixture (50 mM Tris, pH 8.0, 75 mM potassium chloride, 3 mM magnesium chloride, 5 mM dithiothreitol, and 500 µM each dATP, dCTP, dGTP, and dTTP) was added. The reaction was incubated at 42°C for 1 h and then held at 4°C. Second-strand cDNA was synthesized by placing 2 µl of first-strand template into a reaction mixture as described above. Thermocycling parameters were optimized and a final profile was employed that consisted of a single cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 48°C, and 3 min at 72°C; and a final extension for 7 min at 72°C. Reactions were resolved by agarose gel (1.4%, wt/vol) electrophoresis in Tris-acetate-EDTA buffer (31). Gels were stained with ethidium bromide and visualized under UV light.

The custom primer combinations generated six overlapping PCR products ranging from approximately 2,000 to 3,500 bp in length. The products were cloned into vector pCR4-Topo using the Topo TA cloning kit (Invitrogen) and transformed into *E. coli* DH10β. Plasmid DNA was purified from transformed cells using alkaline lysis and clones containing PCR product of the expected size (based on primer locations) were identified by digestion with *Eco*RI and visualized by agarose gel electrophoresis. Sequence was obtained using an automated dye termination system (ABI, Inc.) at Washington State University. Additional custom primers were designed to obtain complete nucleotide sequence for each of six clones. The entire PCR product generation and cloning procedure was replicated at least twice, and the products were sequenced at least twice in forward and reverse directions. The 5′ terminus of the NL-3 K viral genome was cloned using the 5′ rapid amplification of cDNA ends (RACE) system (version 2.0; Invitrogen) according to the manufacturer's instructions. Three RACE reactions were conducted and three clones from each reaction were sequenced. Nucleotide and deduced amino acid se-

quence analyses were performed using BLAST (1) and further analyzed using Align Plus (Scientific and Educational Software, Cary, NC).

RESULTS

Host response and serology. Disease reactions of plants included in the HG differential set are detailed in Table 1. Sutter Pink (HG 0) differed significantly in reaction to infection with each strain. Pronounced vein necrosis occurred on primary leaves infected with NL-3 K followed by stunting and severe mosaic at the secondary leaf stage. Plant death occurred 2 to 3 weeks after inoculation with NL-3 K. Less severe necrosis, stunting, and mosaic occurred on Sutter Pink infected with NL-3 D, and the virus could be maintained in the host without resulting in the death of the plant. Stunting and mosaic also occurred in plants infected with RU1 but no necrosis was observed. UI-34 (HG 4) and UI-114-8 (HG 5) infected with NL-3 K expressed severe mosaic symptoms compared with only mild mosaic when infected with NL-3 D. Some bean lines expressed symptoms much earlier when infected with NL-3 K. For example, Black Turtle 1 (HG 8) plants infected with NL-3 K resulted in extensive top necrosis 5 to 7 dpi whereas, at the same time, plants infected with NL-3 D were just beginning to exhibit top necrosis symptoms.

No germ plasm lines containing the resistance genes *bc3* or *I* + *bc3* expressed symptoms after inoculation with NL-3 D or RU1 (Table 2). In contrast, lines USLK-1, USLK-2, USLK-3, USDK-4, and USDK-5 inoculated with NL-3 K resulted in necrotic local lesions, systemic vein necrosis, and chlorotic halos. USCR-8 exhibited a very mild mosaic symptom upon infection with NL-3 K. However, USWK-6, USCR-7, and USCR-9 did not exhibit symptoms after inoculation with NL-3 K. These differential reactions utilizing USLK and USDK lines were useful as a biological assay for distinguishing NL-3 K from NL-3 D.

All cultivars that became symptomatic with NL-3 K reacted positively with the group-specific potyvirus MAb. Plants infected with NL-3 D or NL-3 K also resulted in a positive reaction when tested against the MAb specific to BCMNV but not to the MAb specific for BCMV. Consistent with reactions to NL-3 D, bean cultivars from HGs 6, 7, 11, and 12 did not become systemically infected with NL-3 K, nor was either strain detectable by ELISA in these hosts. Red Kloud (HG 10) was not systemically infected with NL-3 D but exhibited a mild vein necrosis and was ELISA positive when infected with NL-3 K. Lines USLK-1, USLK-2, USLK-3, USDK-4, USDK-5, and USCR 8 inoculated with NL-3 K each reacted positively to the BCMNV MAb but USWK-6, USCR-7, and USCR-9 did not. None of the USLK, USDK, or USCR lines listed above developed systemic infection or reacted positively with the BCMNV group-specific MAb after inoculation with NL-3 D, nor did they react positively with the BCMV group-specific MAb.

Sequence analysis. The complete nucleotide sequence of the NL-3 K genome (AY864314) was 9,893 nucleotides (nt) in length compared with 9,626 nt for NL-3 D. The deduced amino acid sequence of NL-3 K was a single polyprotein similar in structure to the Washington isolate of NL-3 D (AAN27999) and other members of the family *Potyviridae*. The predicted molecular weight of the polyprotein was 362.1 kDa for NL-3 K compared with 350.9 kDa for NL-3 D. The 5′ UTR of NL-3 K consisted of 140 nt that shared 96% identity with that of RU1. In contrast, the 5′ UTR of NL3-D was 169 nt in length and only 59% identical with the same region of NL-3 K. The 3′ UTR extended 245 nt downstream and was 100% identical with the 3′ UTR of NL-3 D (AY138897).

Predicted CP amino acid sequences of NL-3 K and NL-3 D shared 99% identity. Only two amino acid differences were noted. The first substitution occurred at the 5′ end of the CP gene located just upstream of the DAG motif typically associated with aphid transmissibility (2). At amino acid position 6 of the CP, the glutamic acid residue in NL-3 D was substituted by an alanine (SSKKEAEK) residue in NL-3 K. The second substitution occurred at position 217, where the arginine residue in NL-3 D also was replaced with an alanine residue in NL-3 K. However, when compared with the amino acid sequence of a Michigan isolate (AAB02170) of NL-3 D described by Fang et al. (7), the CP of NL-3 K was 100% identical. Other amino acid substitutions within the genome are detailed in Table 3.

TABLE 2. Differentiation of the RU1 strain of *Bean common mosaic virus* and the NL-3 D and NL-3 K strains of *Bean common mosaic necrosis virus* by symptom expression across a set of selected bean germplasm lines

Germ plasm	Host genes	RU1	NL-3 D	NL-3 K
USLK-1	*I*, *bc-3*	ns	ns	NLL, sVN, Chl
USLK-2	*I*, *bc-3*	ns	ns	NLL, sVN, Chl
USLK-3	*I*, *bc-3*	ns	ns	NLL, sVN, Chl
USDK-4	*I*, *bc-3*	ns	ns	NLL, sVN, Chl
USDK-5	*I*, *bc-3*	ns	ns	NLL, sVN, Chl
USWK-6	*I*, *bc-3*	ns	ns	ns
USCR-7	*I*, *bc-3*	ns	ns	ns
USCR-8	*bc-3*	ns	ns	mM
USCR-9	*I*, *bc-3*	ns	ns	ns

[a] Chl = chlorosis; mM = mild mosaic; NLL = necrotic local lesions; ns = no symptoms; sVN = systemic vein necrosis.

TABLE 1. Reactions of bean host group differentials to the RU1 strain of BCMV and the NL-3 D and NL-3 K strains of BCMNV at 11 and 19 days post-inoculation (dpi)[a]

			RU1		NL-3 D		NL-3 K	
Host differential	HG	Resistance genes	11 dpi	19 dpi	11 dpi	19 dpi	11 dpi	19 dpi
Sutter Pink	0	None	ns	st, M	Chl	st, sM	Chl, VN	sM, st, pd
Bountiful	1	*bc-u*	ns	st, M	Chl	sM	mChl	sM
Redlands Greenleaf C	2	*bc-1*	ns	Mot	Chl	M	Chl	M
Redlands Greenleaf B	3	*bc-1*^{2}	ns	Mot	Chl	mM	Chl	M
UI-34	4	*bc-2*	ns	M	Chl, VN	M	Chl, VN	sM
UI-114-8	5	*bc-1*, *bc-2*	ns	ns	sChl	mM	mChl	sM
UI-31	6	*bc-1*^{2}, *bc-2*^{2}	ns	ns	Chl	ns	mChl	ns
IVT-7214	7	*bc-2*, *bc-3*	ns	ns	ns	ns	ns	ns
Black Turtle 1	8	*I*	ns	ns	VN	TN	VN	TN
Top Crop	9	*I*, *bc-1*	ns	ns	VN	TN	VN	TN
Jubila	9	*I*, *bc-1*	ns	ns	VN	VN1	VN	ns
Red Kloud	10	*I*, *bc-1*^{2}	ns	ns	VN	ns	VN	VN1
US92-1006	11	*I*, *bc-2*^{2}	ns	ns	NLL	ns	NLL	ns
TARS-VR-7s	12	*I*, *bc-3*	ns	ns	ns	ns	ns	ns

[a] HG = host group; primary and secondary reactions were observed 11 and 19 dpi, respectively; Chl = chlorosis; M = mosaic; mChl = mild chlorosis; mM = mild mosaic; Mot = mottle; NLL = necrotic local lesions; ns = no symptoms; pd = plant death; sChl = severe chlorosis; sM=severe mosaic; st = stunted growth; TN = top (systemic) necrosis; VN = vein necrosis; VN1 = vein necrosis in one or two patches on the first trifoliate leaf.

The P1 protein of NL-3 K consisted of 415 amino acids compared with 317 amino acids for NL-3 D. When compared against available virus sequences in GenBank using the BLAST algorithm (1), the first 114 predicted amino acids of the NL-3 K P1 region shared a 98% identity with BCMV strain RU1. The remaining 301 amino acids of the NL-3 K P1 protein shared only 34% identity with RU1 but shared 98% identity with NL-3 D. A glutamine residue at position 115 in NL-3 K that was not present in NL-3 D served as the transition point representing the putative recombination event between BCMV-RU1 and NL-3 D to form the new NL-3 K strain. An amino acid alignment schematic of the P1 protein of NL-3 D, RU1, and NL-3 K is presented in Figure 1. Comparison of the same predicted 114 amino acids of NL-3 K with other virus strains using BLAST indicated a more distant relationship (85 to 87% identity) with the blackeye cowpea strain of BCMV. The least-related (12% identity) potyvirus recognized by the BLAST search was *Onion yellow dwarf virus* (NP871738).

TABLE 3. Summary of amino acid sequence comparisons between NL-3 K and NL-3 D

Gene[a]	Amino acids	Identity (%) with NL-3 D	Amino acid substitutions	Substitution position[b]
P1	415	See text	See text	See text
HC-Pro	408	99	2	455, 786
P3	405	99	3	1204, 1215, 1216
6K1	52	98	1	1263
CI	634	99	1	1624
6K2	53	94	3	1907, 1912, 1952
VPG	190	100	0	…
NIa	243	99	1	2324
NIb	517	100	0	…
CP	261	99	2	2914, 3125

[a] Specific protein genes were determined according to Fang et al. (7). HC-Pro = helper component-proteinase and CP = coat protein.
[b] Indicates position location on polyprotein.

RT-PCR. Primers designed for NL-3 K, NL-3 D, and RU1 each produced amplicons of the expected size from plants infected with each virus (Fig. 2). To demonstrate that NL-3 K was a unique recombinant virus strain and not a mixed infection of the two viruses, primers were designed that flanked the point of recombination. The forward primer (5′-ATGGACACTACCACTGTCAC-3′) was designed using the obtained NL-3 K sequence and was located 311 bases downstream of the 5′ terminus, whereas the complimentary primer (5′-AGTTCACCGTGAGATGTC-3′) was the same used for NL-3 D and was located at nucleotide 1157. RT-PCR reactions with these new primers using total nucleic acid preparations from infected bean plants as template produced a single amplification product of the expected size (847 bp) for the NL-3 K strain that was not produced from the NL-3 D, RU1, or healthy bean templates (Fig. 2). Sequence analysis verified

```
RU1     (  1) .....................................P..........
NL-3K   (  1) MASIMIGTITVPLVWNECTYKEVGEFIEVETQERVYMNSKPTSARKRNLC
NL-3D   (  1) ..T..F.S.----------------------------------------

RU1     ( 51) ............Y.....................................
NL-3K   ( 51) FCDCDDDGHYHCHFCDCECDSKNHLEEHERDVCEDAYSVRAFGYKLVPKI
NL-3D   ( 10) ------------------------------------------------

RU1     (101) ..............P.T.SL.---L..VKNN.CEITAQTKVASNI.TKDM
NL-3K   (101) EIKRITKKVPIAKSQEAIVIAMPKSKHTLHVVQ------------VEAKH
NL-3D   ( 10) -------AAE.PVIK...M.............------------.....

RU1     (148) ..KSEP.LKQVNRALVLVGRKEVDN..LTV.KM.EAMQQ..V..R...MQ
NL-3K   (139) MATEIRSERGKLYVAKRFADNAIKAYDSQLKAFDGLLKKNSDLQKRLFIG
NL-3D   ( 41) .................................E..............

RU1     (198) .Q.T...QPK..VQLSLC.YE..K..VKLTR.KYG..KA..Q.K.EQ.E.
NL-3K   (189) QNSPIKQKKGGACFVRSLSFKQAEERHAKYLKLQEEEHQFLSGAYGDKVY
NL-3D   ( 91) .............................................A.

RU1     (248) I.K.LEPIVIQRGQSIG.....WH.SF.KSINNPPKR.VEPPTR..REI.
NL-3K   (239) VGSVQGTLDRKVAEKVSFKSPYYKRTCKAVRQVKVLKKAVGSGKVLDQVL
NL-3D   (141) ................................................

RU1     (298) NVIRDK..SIE.I.R.-T.R.T.R..NKGNS.V..VI.P.....YKKK.L
NL-3K   (289) EIVAETGVPVTFVGKGANKTLRAQYVRRYGLVIPKIFLCHESGRKVHREM
NL-3D   (191) ................................................

RU1     (347) DINIY.QC.AA..A..T.RH..NGEIKP.....V..K.SSLTADH.QQ.F
NL-3K   (339) SYCHHKETLQYLCKHGKYGALNENALCKGDSGLLFDQRTAFVKRVTYLPH
NL-3D   (241) ..W.............................................

RU1     (397) M.I...LD.E..N.LDEQQDIYS.H..
NL-3K   (389) FIVRGRQEGQLVCATEYLDNVHTIEHY
NL-3D   (291) ...................Y.....
```

Fig. 1. P1 protein amino acid sequence comparison of the P1 protein region of *Bean common mosaic virus* strain RU1, *Bean common mosaic necrosis virus* (BCMNV) strain NL-3 K, and BCMNV strain NL-3 D. The glutamine (Q) residue in shaded box represents the recombination point occurring in NL-3 K.

authenticity of the NL-3 K amplicon which was identical in sequence to the equivalent region in the NL-3 K P1 protein. This experiment was repeated several times over the course of this study and produced similar results. Hence, NL-3 K can be differentiated from NL-3 D and RU1 by RT-PCR using strain-specific primers.

DISCUSSION

From all available evidence presented here, NL-3 K appears to be a naturally occurring genomic recombinant between BCMV RU1 and BCMNV NL-3 D. We were able to consistently isolate and identify the point of recombination in NL-3 K using RT-PCR from infected bean plants after weekly serial transfers that occurred over a period of more than 3 years. Sequence data obtained from cloned RT-PCR amplicons in the region near the recombination event were 100% identical in all replications. These data, together with the consistent biological responses observed in this study and by others (37), provide strong evidence that the recombinant NL-3 K genome is stable in vivo.

Previous reports have linked the HC-Pro region of potyviruses to pathogenicity and symptom expression (36,40). Although it has been suggested that the P1 protein may be involved in virus–host interactions of potyviruses (33), less evidence is available demonstrating the role of P1 on pathogenicity and host specificity. For example, Saenz et al. (29) demonstrated that sequence variation in the P1 region was not responsible for symptom differences between two isolates of *Plum pox virus*. In contrast, Klein et al. (12) were able to show that an insertion mutant in P1 of *Tobacco vein mottling virus* induced symptoms ranging from very mild to severe. Desbiez and Lecoq (5) recently demonstrated that a strain of *Watermelon mosaic virus* (WMV) from France was a putative recombinant of WMV and the blackeye cowpea strain of BCMV. The recombinant viral protein consisted of an additional 135 amino acids located at the N terminus of the P1 protein that were 85% identical to BCMV. Interestingly, the NL-3 K strain also encoded additional amino acids located at the 5′ N terminus of the P1 protein. The increased symptom severity on selected common bean hosts induced by NL-3 K suggests that the P1 region may play a significant role in pathogenicity and virulence. By comparison, when Hammond and Hammond (10) compared the complete nucleotide sequence of the gladiolus isolate of *Bean yellow mosaic virus* (BYMV) with isolates BYMV-MB4 and BYMV-S from broad bean, significant variability occurred in the P1 protein as well as other regions of the genome. The authors suggested that the differences in P1 of BYMV-S and the GDD or MB4 strains may be responsible for uncharacteristic symptom expression and variations in host range. Similar results were reported by Zheng et al. (40) with two isolates of BCMV causing different symptoms in asparagus bean where the greatest sequence variation between isolates occurred in the 5′ UTR and the N-terminal of the P1 protein. Although the most significant sequence change in NL-3 K occurred in P1, other minor amino acid substitutions occurred in other regions of the genome compared with that of NL-3 D (Table 3). Although the substitutions in each gene are few, it cannot be discounted that they may play a role in differences in host response.

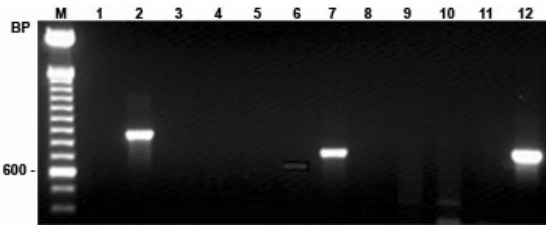

Fig. 2. Agarose gel showing amplification products from reverse-transcriptase polymerase chain reaction of total nucleic acid extracts using primers specific for *Bean common mosaic necrosis virus* (BCMNV) strain NL-3 K (lanes 1 to 4), BCMNV strain NL-3 D (lanes 5 to 8), and *Bean common mosaic virus* strain RU1 (lanes 9 to 12) in common bean (*Phaseolus vulgaris* L.). Lane M, 100-bp marker; lanes 1, 5, and 9, extracts from healthy bean control; lanes 2, 6, and 10, extracts from bean infected with NL-3 K; lanes 3, 7, and 11, extracts from bean infected with NL-3 D; and lanes 4, 8, and 12, extracts from bean infected with RU1.

The geographic origin of the putative recombinant between RU1 and NL-3 is unknown, because there is limited information available regarding the history of RU1. The RU1 strain first was identified in 1985 in bean seed from a United States Department of Agriculture Plant Introduction accession from Russia (14,34). To our knowledge, RU1 never was identified in fields in Washington or Idaho, nor was it ever included as a test strain for resistance screening of common bean lines. The virus was determined to be a strain of BCMV (serotype B) based on serology (24) and CP sequence (GenBank) comparison with other strains of BCMV. McKern et al. (14) originally had placed RU1 in pathogenicity group (pathogroup) VII. RU1 later was placed in pathogroup VI, according to host responses in the bean differential group developed by Drijfhout (6,34) and verified under our conditions (data not shown). Prior to the discovery of RU1, pathogroup VI was assigned only to serotype A, consisting of BCMNV strains NL-3 (D) and NL-5, and involved three pathogenicity genes (pathogenes), including P1, $P1^2$, and P2 (6). RU1 is currently the only strain of BCMV also assigned to pathogroup VI and the only BCMV strain to share a pathogroup typically associated with serotype A (BCMNV) viruses. Accordingly, the pathogenicity of RU1 involves the same pathogenes P1, $P1^2$, and P2 as NL-3 D. Thus, the change in pathogenicity as indicated by ability of NL-3 K (but not NL-3 D or RU1) to infect USLK-1, USLK-2, USLK-3, USLK-4, and USCR-8 as a result of putative recombination between RUI and NL-3 D was not predicted. These results provide additional evidence that pathogenicity may be linked to the P1 protein gene. It is reasonable to argue that virulence also may be linked to the P1 gene, because symptoms caused by NL-3 K occurred earlier and were more severe in selected lines containing the *I* and bc^3 genes compared with the same lines infected with NL-3 D. Silbernagel et al. (34) hypothesized that recombination among strains of BCMV and BCMNV was possible based on viral phenotypes. Although apparent recombination occurred in their experiments, a stable genomic recombinant between NL-8 (BCMNV) and US-5 (BCMV) representing pathogroups III (serogroup A) and IV (serogroup B), respectively, never was generated successfully. It is plausible that a stable genomic recombination between NL-3 and RU1 resulting in the NL-3 K strain may have occurred because of similar pathogenes present in the two parental viral genotypes.

The differential resistance response to NL-3 K among *I* + *bc-3* bean genotypes USLK-1, USLK-2, USLK-3, USDK-4, and USDK-5, as well as USWK-6, USCR-7, and USCR-9, was unexpected. In a preliminary study (P. N. Miklas, *unpublished data*), the high resistance in USWK-6, USCR-7, and USCR-9 was conditioned by a single recessive gene and appeared to be allelic to the reduced levels of resistance in USLK-1, USLK-2, USLK-3, USDK-4, and USDK-5. The highly resistant *I* + *bc-3* genotypes may have an additional gene or allele conferring a greater level of resistance. Conversely, *I* + *bc-3* genotypes USLK-1, USLK-2, USLK-3, USDK-4, and USDK-5 may possess a less effective allele at *bc-3*, resulting in a breakdown of resistance when inoculated with NL-3 K. Further investigation of these lines is necessary to explore additional sources of resistance to BCMNV and other related potyviruses.

ACKNOWLEDGMENTS

Mention of proprietary or brand names are necessary to report factually on available data; however, the U.S. Department of Agriculture

(USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval to the exclusion of others that also may be suitable. We thank K. C. Eastwell for excellent editorial contributions.

LITERATURE CITED

1. Altschul, S. F., Madden, T. L., and Schäffer, A. A. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
2. Atreya, P. L., Atreya, C. D., and Pirone, T. P. 1991. Amino acid substitutions in the coat protein result in loss of insect transmissibililty of a plant virus. Proc. Natl. Acad. Sci. USA 88:7887-7891.
3. Berger, P. H., Wyatt, S. D., Shiel, P. J., Silbernagel, M. J., Druffel, K., and Mink, M. I. 1997. Phylogenetic analysis of the *Potyviridae* with emphasis on legume-infecting potyviruses. Arch. Virol. 142:1979-1999.
4. Cervera, M. T., Riechmann, J. L., Martin, M. T., and Garcia, J. A. 1993. 3′-Terminal sequence of the plum pox virus PS and o6 isolates: Evidence for RNA recombination within the potyvirus group. J. Gen. Virol. 74:329-334.
5. Desbiez, C., and Lecoq, H. 2004. The nucleotide sequence of *Watermelon mosaic virus* (WMV, *Potyvirus*) reveals interspecific recombination between two related potyviruses in the 5′ part of the genome. Arch Virol. 149:1619-1632.
6. Drifjhout, E. 1978. Genetic Interaction Between *Phaseolus vulgaris* and Bean common mosaic virus with Implications for Strain Identification and Breeding for Resistance. Centre for Agricultural Publication and Documents, Wageningen, The Netherlands.
7. Fang, G. W., Allison, R. F., Zambolim, E. M., Maxwell, D. P., and Gilbertson, R. L. 1995. The complete nucleotide sequence and genome organization of bean common mosaic virus (NL3 strain). Virus Res. 39:13-23.
8. Flores-Estevez, N., Acosta-Gallegos, J. A., and Silva-Rosales, L. 2003. *Bean common mosaic virus* and *Bean common mosaic necrosis virus* in Mexico. Plant Dis. 87:21-25.
9. Glais, L., Tribodet, M., and Kerlan, C. 2002. Genomic variability in *Potato potyvirus Y* (PVY): Evidence that PVY(N)W and PVY(NTN) variants are single to multiple recombinants between PVY(O) and PVY(N) isolates. Arch. Virol. 147:363-378.
10. Hammond, J., and Hammond, R. W. 2003. The complete nucleotide sequence of isolate BYMV-GDD of *Bean yellow mosaic virus*, and comparison to other potyviruses. Arch. Virol. 148:2461-2470.
11. Kelly, J. D. 1997. A review of varietal response to bean common mosaic potyvirus in *Phaseolus vulgaris*. Plant Var. Seeds 10:1-6.
12. Klein, P. G., Klein, R. R., Rodriguez-Cerezo, E., Hunt, A. G., and Shaw, J. G. 1994. Mutational analysis of the tobacco vein mottling virus genome. Virology 204:759-769.
13. Larsen, R. C., Kaiser, W. J., Wyatt, S. D., Buxton-Druffel, K. L., and Berger, P. H. 2003. Characterization of a new potyvirus naturally infecting chickpea. Plant Dis. 87:1366-1371.
14. McKern, N. M., Mink, G. I., Barnett, O. W., Mishra, A., Whittaker, L. A., Silbernagel, M. J., Ward, C. W., and Shukla, D. D. 1992. Isolates of Bean common mosaic virus comprising two distinct potyviruses. Phytopathology 82:923-929.
15. McKern, N. M., Ward, C. W., and Shukla, D. D. 1992. Strains of bean common mosaic virus consist of at least two distinct potyviruses. Arch. Virol. Suppl. 5:407-414.
16. Miklas, P. N., and Hang, A. N. 1998. Release of light red kidney dry bean germplasm lines USLK-1, -2, and -3 with resistance to bean common mosaic and necrosis viruses. Annu. Rep. Bean Improv. Coop. 41:231-232.
17. Miklas, P. N., and Hang, A. N. 1998. Release of dark red and white kidney dry bean germplasm lines USDK-4, USDK-5, and USWK-6 with resistance to bean common mosaic and necrosis viruses. Annu. Rep. Bean Improv. Coop. 41:229-230.
18. Miklas, P. N., and Hang, A. N. 1998. Release of cranberry dry bean germplasm lines USCR-7 and USCR-8 with resistance to bean common mosaic and necrosis viruses. Annu. Rep. Bean Improv. Coop. 41:227-228.
19. Miklas, P. N., Hang, A. N., Kelly, J. D., Strausbaugh, C. A., and Forster, R. L. 2002. Registration of three kidney bean germplasm lines resistant to *bean common mosaic* and *necrosis potyviruses*: USLK-2 light red kidney, USDK-4 dark red kidney, and USWK-6 white kidney. Crop Sci. 42:674-675.
20. Miklas, P. N., and Kelley, J. D. 2002. Registration of two cranberry bean germplasm lines resistant to *bean common mosaic* and *necrosis potyviruses*: USCR-7 and USCR-9. Crop Sci. 42:673-674.
21. Miklas, P. N., Strausbaugh, C. A., Larsen, R. C., and Forster, R. L. 2000. NL-3 (K)—A more virulent strain of NL-3 and its interaction with *bc-3*. Annu. Rep. Bean Improv. Coop. 43:168-169.
22. Mink, G. I., and Silbernagel, M. J. 1992. Serological and biological relationships among viruses in the bean common mosaic virus subgroup. Arch. Virol. Suppl. 5:397-406.
23. Mink, G. I., Vetten, J., Ward, C. W., Berger, P. H., Morales, F., Meyers, J. R., Silbernagel, M. J., and Barnett, O. W. 1994. Taxonomy and classification of legume-infecting potyviruses: A proposal from the *Potyviridae* study group of the plant virus subcommittee of ICTV. Arch. Virol. 139:231-235.
24. Mink, G. I., Vetten, H. J., Wyatt, S. D., Berger, P. H., and Silbernagel, M. J. 1999. Three epitopes located on the coat protein amino terminus of viruses in the bean common mosaic potyvirus subgroup. Arch. Virol. 144:1173-1189.
25. Moravec, T., Cerovska, N., and Boonham, N. 2002. The detection of recombinant, tuber necrosing isolates of Potato virus Y (PVY(NTN)) using a three-primer PCR based in the coat protein gene. J. Virol. Methods 109:63-68.
26. Ohshima, K., Yamaguchi, Y., Hirota, R., Hamamoto, T., Tomimura, K., Tan, Z., Sano, T., Azuhata, F., Walsh, J. A., Fletcher, J., Chen, J., Gera, A., and Gibbs, A. 2002. Molecular evolution of *Turnip mosaic virus*: Evidence of host adaptation, genetic recombination and geographical spread. J. Gen. Virol. 83:1511-1521.
27. Presting, G. G., Smith, O. P., and Brown, C. R. 1995. Resistance to potato leafroll virus in potato plants transformed with the coat protein gene or with vector control constructs. Phytopathology 85:436-442.
28. Revers, F., Le Gall, O., Candresse, T., Le Romancer, M., and Dunez, J. 1996. Frequent occurrence of recombinant potyvirus isolates. J. Gen. Virol. 77:1953-1965.
29. Saenz, P., Cervera, M. T., Dallot, S., Quiot, L., Quiot, J. B., Riechmann, J. L., and Garcia, J. A. 2000. Identification of a pathogenicity determinant of *Plum pox virus* in the sequence encoding the C-terminal region of protein P3+6K(1). J. Gen. Virol. 81:557-566.
30. Saiz, M., DeBlas, C., Carazo, G., Fresno, J., Romero, J., and Castro, S. 1995. Incidence and characterization of bean common mosaic virus isolates in Spanish bean fields. Plant Dis. 79:79-81.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
32. Sano, Y., van der Vlugt, R., de Haan, P., Takahashi, A., Kawakami, M., Goldbach, R., and Kojima, M. 1992. On the variability of the 3′ terminal sequence of the *Turnip mosaic virus* genome. Arch. Virol. 126:231-238.
33. Shukla, D. D., Ward, C. W., and Brunt, A. A. 2004. The *Potyviridae*. CAB International, Wallingford, UK.
34. Silbernagel, M. J., Mink, G. I., Zhao, R. L., and Zheng, G.-Y. 2001. Phenotypic recombination between bean common mosaic and bean common mosaic necrosis potyviruses in vivo. Arch. Virol. 146:1007-1020.
35. Spence, N. J., and Walkey, D. G. A. 1995. Variation for pathogenicity among isolates of bean common mosaic virus in Africa and a reinterpretation of the genetic relationship between cultivars of *Phaseolus vulgaris* and pathotypes of BCMV. Plant Pathol. 44:527-546.
36. Stenger, D. C., and French, R. 2004. Functional replacement of *Wheat streak mosaic virus* HC-Pro with the corresponding cistron from a diverse array of viruses in the family *Potyviridae*. Virology 232:257-267.
37. Strausbaugh, C. A., Miklas, P. N., Singh, S. P., Myers, J. R., and Forster, R. L. 2003. Genetic characterization of differential reactions among host group 3 common bean cultivars to NL-3 K strain of *Bean common mosaic necrosis virus*. Phytopathology 93:683-690.
38. Tan, Z., Wada, Y., Chen, J., and Ohshima, K. 2004. Inter- and intralineage recombinants are common in natural populations of *Turnip mosaic virus*. J. Gen. Virol. 85:2683-2696.
39. Vetten, H. J., Lesemann, D. E., and Maiss, E. 1992. Serotype A and B strains of bean common mosaic virus are two distinct potyviruses. Arch. Virol. Suppl. 5:415-431.
40. Zheng, H., Chen, J., Chen, J., Adams, M. J., and Hou, M. 2002. *Bean common mosaic virus* isolates causing different symptoms in asparagus bean in China differ greatly in the 5′-parts of their genomes. Arch. Virol. 147:1257-1262.